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INFLAMMATORY CELLS IN ADJUVANT ARTHRITIS

BY

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Bone Marrow as the Source of Inflammatory Cells in Adjuvant Arthritis

Introduction

The injection of mycobacterial adjuvant into rats induces in approximately 10 days a symmetrical migratory polyarthritis which increases in severity for about 2 weeks and is variably associated with iridocyclitis, subcutaneous nodules and mucocutaneous lesions[1-3]. Injection of oil alone or oil with a variety of other bacteria or irritants does not produce the disease[2]. A latent period of about 10-16 days exists between sensitization and clinical onset[1,2,4,5]. Sensitization is most effective via intradermal injection, especially in the footpad, whereas subcutaneous, intravenous, and intraperitoneal routes are relatively ineffective[4]. Attempts to culture bacteria or PPLO have been unsuccessful and administration of various antibiotics before and during the latency period have no effect on the development of the disease[2,6]. Attempts to transfer the disease with diseased tissue, serum, or killed sensitized lymph node cells have failed; however, the disease can be transferred by living sensitized lymphoid cells to recipient rats of the same inbred strain[4,6-8]. It has been impossible to

induce the disease in rats less than 5 weeks old[2,4]. A shortening of the latency period is noted in animals reinoculated after having previously had the disease[2,4]. Administration of corticosteroids or high doses of x-irradiation (600r but not 200r) markedly modify the incidence and severity of the arthritis[2,4]. All this evidence suggests that this disease is a delayed hypersensitivity response to a disseminated antigen, the exact nature of which is still not known but is thought to be a constituent of the injected tubercle bacilli. Histologically, also, adjuvant arthritis lesions resemble lesions of delayed hypersensitivity[2,5,9].

The early lesions of adjuvant arthritis are similar regardless of location. They consist of congested small vessels and perivascular infiltration consisting principally of medium-sized lymphocytes[5]. As the lesions progress, the infiltrate becomes more diffuse. Monocytes and histiocytes predominate in lesions of greater than 2-3 days' duration. Fibroblastic proliferation may be marked in later lesions, as is synoviocyte proliferation in joint and tendon lesions. Also in the older lesions there may be a diffuse infiltrate of polymorphonuclears or even foci of necrosis. These foci of necrosis occur primarily in large lesions at the center of an area of histiocyte infiltration surrounded by an area of lymphocyte infiltration. As the clinical disease progresses,

there is an increase in the number of lesions was well as an increase in their size. New lesions develop at different sites and the age of the various lesions may differ in the same area.

Autoradiographic evidence using tritiated thymidine labeling shows that the infiltrating mononuclears are hematogenous and derived from an undefined rapidly dividing precursor population[10]. It appears that the histiocytes, which are the predominant cell type in older lesions, are derived at least in part from the hematogenous medium-sized lymphocytes, which continue to divide once they reach the lesion itself. These results[10] closely parallel the results of other tritiated thymidine labeling experiments in delayed skin reactions[11], autoallergic encephalomyelitis[12], and thyroiditis[13], and add to evidence which suggests a close similarity between adjuvant arthritis and these reactions of delayed hypersensitivity.

McCluskey et al[14], combining passive transfer with tritiated thymidine labeling, have established that only a small number of the infiltrating cells in delayed skin reactions in the recipients are actually sensitized cells. Their experiments failed to show any specific attraction of the sensitized cells for the test site. Others have found similar results[15-17]. Only 2 studies[18,19]

have shown an apparent specificity of the sensitized cells for the specific test site. Since the numbers of sensitized cells in the reaction sites are so low, the question of specificity appears undecided. The effector cell in initiating the reaction of delayed hypersensitivity, however, seems fairly certain to be the sensitized lymphocyte, since as few as 2% sensitized lymphocytes with 98% normal macrophages behave as if all the cells were sensitized in in vitro experiments[20].

Volkman and Gowans[21-23] have shown using tritiated thymidine labeled cells that the macrophages of non-specific inflammatory exudates must have precursors that release their progeny into the blood at other sites than the area of inflammation. To determine the source of precursors of the macrophages, they removed their various possible sources by splenectomy, thymectomy, lymphocyte depletion and total body x-irradiation. They found that both the migration and the labeling of macrophages were suppressed by 750r of x-irradiation and in turn restored by marrow shielding during irradiation. Lidén[24] has used tritiated thymidine labeling to show that the infiltrating cells in allergic contact dermatitis also are derived from bone marrow precursors. Boak et al[25] using the T^6 chromosomal marker have found similar results in the liver lesions of graft-versus-host

disease, another delayed hypersensitivity reaction. Using fluorescence labeling combined with passive transfer, Lubaroff and Waksman[26-28] have shown that the majority of the infiltrating mononuclear cells in both nonspecific inflammatory reactions induced by turpentine and in delayed tuberculin skin reactions are also derived from the bone marrow. My experiment was designed to use the same technique to investigate the possibility that the infiltrating cells in the joint lesions of adjuvant arthritis are also derived from bone marrow precursors.

Materials and Methods

Animals. Male and female rats of the inbred Lewis and BN strains were obtained from Microbiological Associates, Inc., of Walkersville, Md. F_1 hybrids of (BNxL) crosses were bred in our own labs. Rats were kept in cages in groups of 6 or less (with the exception of suckling F_1 hybrids), fed standard laboratory chow, and watered ad libitum.

Thymectomy. Male Lewis rats were thymectomized at 5-6 weeks of age. Ether was used for anesthesia. The thorax was shaved, cleaned with 70% alcohol, a midline incision made and the strap muscles separated. The

anterior two-thirds of the sternum was transected and retracted, the thymus lobes gently pulled free, and the chest closed with 5-0 silk and the skin with auto-clips. If the rat had sustained a pneumothorax, artificial respiration was given.

X-irradiation. 21-26 days after thymectomy, each rat received a central axis dose of 900r total body x-irradiation. They were placed in a 3-cell lucite cage and rotated under a 250 KV Westinghouse machine set at 15mA using a 2mm aluminum filter and 0.8mm copper half-value layer. The dose rate was 147.3 rads per minute with the focus-to-skin distance set at 54cm. The dorsal surface received 1125r and the ventral surface 675r[29].

Transfer of normal bone marrow cells. 2-4 month old Lewis or F_1 rats were used as bone marrow donors for the irradiated Lewis rats. Marrow cells were washed from the humeri, femurs, and tibias with, and suspended in, Hanks' Balanced Salt Solution(HBSS)*. The cells were counted, spun at 1000 rpm in a refrigerated centrifuge** at $4^{\circ}C$ for 10 minutes, and resuspended in 4ml of HBSS to give from 2.0×10^8 to 4.2×10^8 nucleated

* obtained from Grand Island Biologicals Co, Grand Island, NY.

**International Equipment Co, Model PR-2.

cells to each recipient. The cells were injected intravenously the day of irradiation.

Passive transfer of adjuvant arthritis with lymph node cells. 10-15 week old male Lewis rats were injected with 0.1 ml in each foot of a 3 mg/ml suspension of finely ground heat-killed tubercle bacilli in oil*. 9 days after sensitization, these animals were sacrificed with ether. The popliteal, inferior epigastric, subscapular, axillary, cervical, mesenteric and para-aortic nodes were harvested, cleaned of adventitious tissue and squeezed through stainless steel mesh in a garlic press with HBSS. The cells were counted in a hemocytometer using 3% acetic acid as a diluent. Trypan blue was used to determine the percentage of viable cells. The cells were centrifuged 10 minutes as above and re-suspended in 4ml of HBSS. Two donors were used per recipient, giving $6.7x10^8$ to $12.0x10^8$ viable cells for intravenous injection. Transfers were carried out on the same day as x-irradiation and bone marrow reconstitution with the exception of one of the preliminary experiments in which the transfers were carried out 7 days after irradiation and bone marrow transfer.

Clinical observations. The recipients were examined

*Bayol F, Humble Refining Co.

daily for clinical onset of the passive disease, and then daily for its severity. Onset was taken to be the first appearance of redness and swelling of any of the distal joints of the extremities or the appearance of ear nodules. The lesions were graded on an arbitrary scale of + to ++++ with increasing severity, + being just detectable and ++++ representing a functionally incapacitated joint. Figures 1. and 2. show typical lesions of passive adjuvant disease.

Fluorescence assay. The indirect fluorescent antibody technique originally described by Möller[30] for use in demonstrating mouse isoantigens and adapted by Lubaroff[29] for the use of rat histocompatibility antigens as cell markers was used.

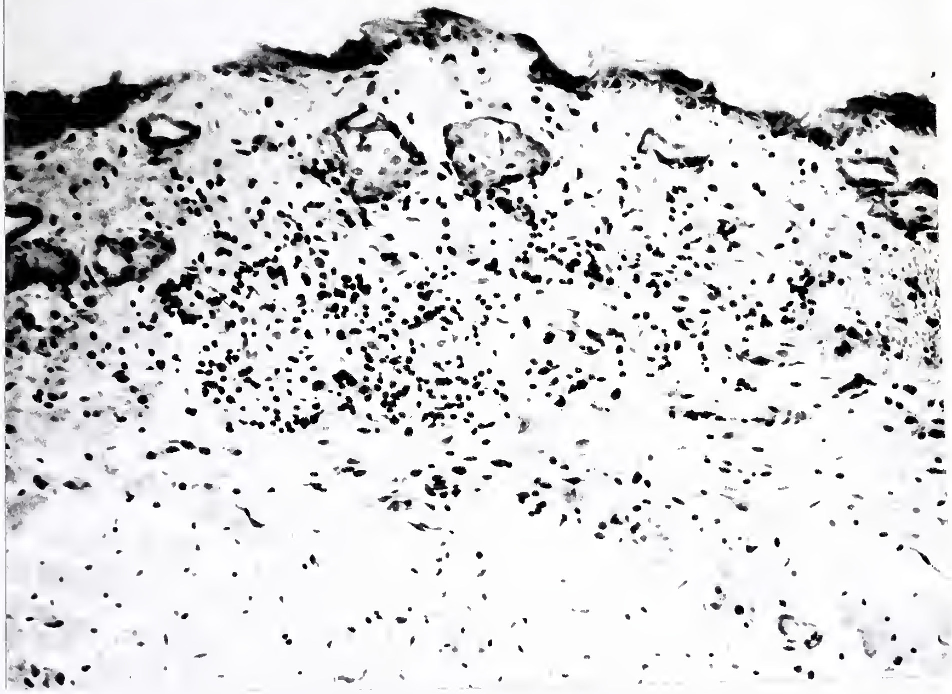
Preparation of cells. Thymectomized, x-irradiated, Lewis rats which had received F_1 marrow and sensitized lymph node cells and which had developed the passive disease were sacrificed at 9-13 days after transfer. Live cell suspensions from the affected joints, the bone marrow, spleen and lymph nodes were prepared. Cells from affected joints were obtained by vigorous teasing with a tissue rake, from the bone marrow by repeated washings of the femurs, and from the spleen and lymph nodes by pressing them through stainless steel mesh, all using HBSS. The cell suspensions

Figure 1 A. Typical affected joints in left hind foot in thymectomized, x-irradiated Lewis rat repopulated with syngeneic bone marrow cells. Photo taken at 5 days after x-irradiation and transfer, intensity graded ++.

Figure 1 B.

Figure 1 C.

Figure 1 B & C. Other views of the same rat as Figure 1 A on the same day.



2A.

2 B.

Figure 2A & B. Inflammatory lesions in thymectomized, x-irradiated Lewis rat repopulated with F_1 hybrid bone marrow and sacrificed for assay 9 days after irradiation and transfer. Hematoxylin and eosin sections of ear nodule (A) and fore-foot m-p joint (B), x 125.

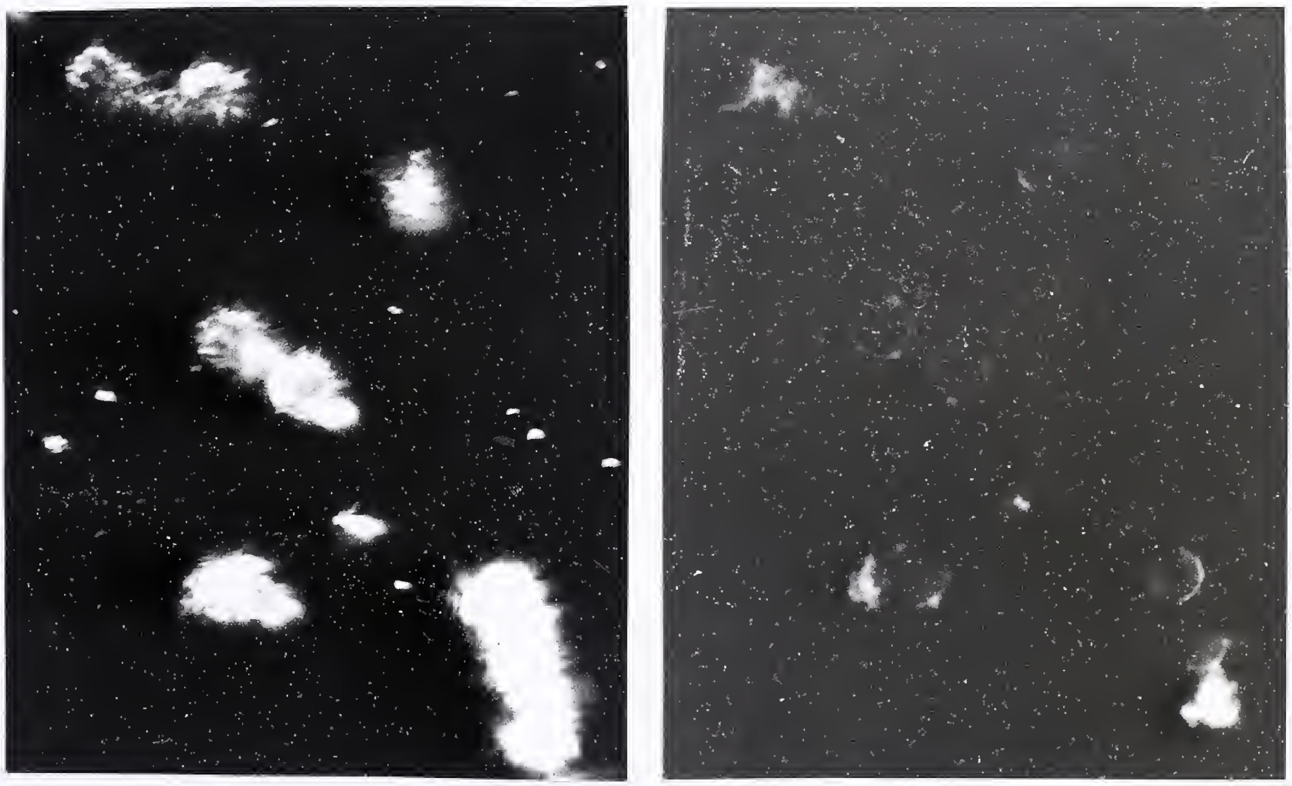
were counted, spun at 1000 rpm in the PR-2 centrifuge at 4°C for 10 minutes and resuspended in HBSS to give a final concentration of 10^7 cells/ml, of which 1ml aliquots were centrifuged as before. The cells were resuspended in 0.5ml of Lewis isoantiserum against BN (see below) and incubated at room temperature for 15 minutes, then centrifuged as above and washed twice with HBSS. They were next incubated 15 minutes at room temperature with 0.1ml of fluorescein-conjugated rabbit antiserum against rat γ-globulin*, centrifuged again and washed twice with HBSS. The cells were finally resuspended in 0.5ml HBSS and placed in an ice bath until examined under the fluorescence microscope.

Preparation of Lewis anti-BN antisera. BN spleen and lymph nodes were harvested and suspensions prepared as described above for sensitized lymph nodes. From 4×10^8 to 5×10^8 cells suspended in HBSS were injected intraperitoneally into adult Lewis rats. This procedure was repeated every 14 days for a total of 3 injections. The rats were bled 6 days after the last injection. The clot was left at 4°C overnight, and the serum separated and heat inactivated at 56°C for

*a commercial preparation obtained from Microbiological Associates, Inc., Bethesda, Md.

30 minutes the following day. The antiserum was then frozen for later use in aliquots which were diluted 1:6 with HBSS and refrozen for use in fluorescent assays. Absorption of the antisera was not necessary, since there is a negligible cytotoxic effect which is due to a very low concentration of BN antigen on the surface of the (BNxL) F_1 hybrid cells[29].

Microscopy. The live cell preparations were examined using a dark field condenser on a Zeiss photomicroscope fitted with 2 light sources, a tungsten lamp and a Sylvania HGK-200 ultraviolet lamp. A 3mm thick BG12 exciter filter and numbers 53 and 47 barrier filters were used for fluorescence microscopy. A drop of the cell suspension was placed on a clean slide, a cover slip placed, and the nucleated cells counted first under dark field illumination and then the number of them which fluoresced with the UV light determined. At least 100 white cells were counted for each tissue, and the percentages of fluorescent cells determined. Control cell preparations of F_1 bone marrow and Lewis bone marrow were examined with each assay as positive and negative fluorescence controls respectively. Figure 3. shows a typical group of cells under dark field and under ultraviolet illumination.



3A. 3B.

Figure 3A & B. Typical lymph node cells under dark field (A) and ultraviolet (B) illumination, x 700. F_1 hybrid control.

Results

1. Adjuvant arthritis - active disease.

In a preliminary experiment, 6 normal Lewis rats 10-11 weeks of age were actively sensitized to determine onset and intensity of the active disease for comparison with the onset and intensity of the passive disease. Onset occurred at 9 days after sensitization for 3 animals, 10 days for 1, and 11 days for 2. Intensity increased over approximately a week after onset until all extremities in all the rats were maximally affected and they could not walk. They were not observed over a long term to determine recovery.

2. Passive transfer of adjuvant arthritis.

In another preliminary experiment, passive transfer of adjuvant arthritis was carried out in normal Lewis recipients in order to determine the number of sensitized lymph node cells necessary to produce the passive idsease as well as to give a standard of passive disease including clinical onset and intensity with which to compare the passive disease in the more complex recipients used later. Doses of 6.0×10^8 viable sensitized lymph node cells and less were found to be ineffective in inducing the passive disease. Onset occurred at 3-7 days and intensity varied from + to ++++ (Table 1).

Table I. Passive Transfer of Adjuvant Arthritis in Normal Lewis Rats.

Rat #	No. of viable, sensitized lymph node cells received ($\times 10^8$)	Onset of arthritis (Days after transfer)	Average intensity
1	2.3	—	0
2	2.3	—	0
3	3.4	—	0
4	3.4	—	0
5	5.0	—	0
6	6.0	—	0
7	9.4	4	+++
8	9.6	4	++++
9	9.7	7	++
10	9.9	3	+
11	9.9	5	+++

Although passive transfer of adjuvant arthritis had already been carried out in normal Lewis rats[7,8], it had never been carried out in thymectomized, irradiated animals reconstituted with bone marrow. For this reason, in the last preliminary experiment 11 Lewis rats were thymectomized and later irradiated and given normal syngeneic bone marrow cells (Table 2). Sensitized lymph node cells were given either the same day as irradiation and bone marrow reconstitution or 7 days later. All developed clinical arthritis within 4-7 days, which is in the range found for normal recipients. No difference was found between the group given sensitized lymph node cells the same day as irradiation and bone marrow reconstitution and the group given sensitized lymph node cells 7 days later.

Next, thymectomized, x-irradiated Lewis recipients were given F_1 bone marrow, to prepare them for the indirect fluorescence assay. An earlier study[26] had shown a probable graft-versus-host reaction in similar thymectomized, x-irradiated rats which received bone marrow cells from animals of another inbred strain. This problem was circumvented by use of F_1 hybrid donors which seem to react in a normal physiologic and immunologic manner. They received sensitized lymph node cells the same day as irradiation and bone marrow reconstitution. Again, clinical onset was essentially the same

Table 2. Passive Transfer of Adjuvant Arthritis in Thymectomized, X-irradiated (900r) Lewis Rats Given Syngeneic Bone Marrow.

Rat #	No. of bone marrow cells received ($\times 10^8$)	No. of sensitized lymph node cells received ($\times 10^8$)	Onset of arthritis (day after transfer)	Average intensity
1	3.7	6.7	4	++++
2	3.9	10.8	4	++++
3	2.2	9.0	6	++++
4	3.9	7.3	7	+++
5	2.2	6.7	7	++
6	2.2	7.0	7	+
7*	3.2	9.0	4	+++
8*	3.2	7.2	5	+
9*	2.0	6.7	6	+++
10*	2.0	7.0	6	++

* Passive transfer 7 days after x-irradiation and bone marrow reconstitution.

as in rats of the other 2 earlier groups (Tables 3 and 4), but the intensity was slightly less. Only the x-irradiated rats restored with F_1 bone marrow suffered radiation deaths (33%) and included some animals which failed to develop clinical arthritis within the expected onset period of 3-8 days.

Although the degree of clinical arthritis varied in the recipient rats in all 3 groups, it was not related to the number of sensitized lymph node cells given when the dose given was greater than 6.7×10^8 cells, the minimum dose found effective in inducing passive disease.

3. Immunofluorescence assay of the cellular infiltrate in arthritis joints.

In the 8 animals with clinical arthritis which were examined by the indirect fluorescence antibody technique (Table 5), the percentage of cells containing BN antigen was found to be 42-65% in the joint lesions, 89-96% in the bone marrow, 6-14% in the lymph nodes, and 25-38% in the spleen (Table 6). Thus about half of the cells in the arthritis joints were derived from the infused marrow. Almost all of the bone marrow cells were F_1 type while the spleen and lymph nodes showed a much lower proportion of F_1 marrow-derived cells.

In Giemsa-stained smears of assayed joint cells from 2 rats the percentage of lymphocytes was 55-73%,

Table 3. Passive Transfer of Adjuvant Arthritis in Thymectomized, X-irradiated (900r) Lewis Rats Given F_1 Hybrid Bone Marrow.

Rat #	No. of bone marrow cells ($\times 10^8$)	No. of sensitized lymph node cells ($\times 10^8$)	Onset of arthritis (day after transfer)	Average intensity	Radiation death (day after transfer)
1	4.0	7.8	3	++	
2	4.2	8.9	4	+++	
3	4.0	10.6	4	+++	
4	4.0	8.7	4	++	11
5	4.2	7.3	5	+++	
6	4.0	6.9	5	++	
7	3.2	12.0	5	++	
8	3.8	8.0	5	+	
9	3.8	6.8	6	++	9
10	3.5	7.9	6	++	
11	4.0	9.9	6	++	
12	4.0	9.1	6	+	11
13	4.0	9.0	7	++	
14	4.0	8.0	7	++	
15	4.0	8.0	8	++	
16	4.0	9.7	—	0	8
17.	4.0	7.7	—	0	9
18	4.0	7.9	—	0	13

Table 4. Comparison of Passive Transfer of Adjuvant Arthritis in the Three Experimental Groups of Recipients.

Recipients	No. of rats	Transfused Marrow cells ($\times 10^8$)	Day of transfer after X-ray	Arthritis in Recipients Onset	Arthritis in Recipients Intensity (0-++++)	Radiation Death (per cent)
Normal	5	—	0	3-7	+-++++	0
Thymectomized, 900r, syngeneic marrow	6	2.2-3.9	0	4-7	+-++++	0
	4	2.0-3.0	7	4-6	+-+++	0
Thymectomized, 900r, F_1 marrow	18	3.2-4.2	0	4-8	0-+++ *	33

* 3 rats failed to develop clinical arthritis within the expected 3-8 day latent period.

Table 5. Degree of Clinical Arthritis in Assay Group in Relation to Sensitized Cells Transferred.

Rat #	No. of sensitized lymph node cells transferred ($\times 10^8$)	Onset of arthritis (Days after transfer)	Day of sacrifice	Arthritis at time of assay* Joints involved	Intensity
2	8.9	4	9	4, 1½	+++, ++
5	7.3	5	9	4	+++
6	6.9	5	11	1	++
7	12.0	5	11	2½	++
11	9.9	6	11	2½	++
13	9.0	7	11	2	++
14	8.0	7	11	1½	++
15	8.0	8	13	1, 1	++, +

* Joints include ankle, wrist, or knuckles. Single joints recorded as ½.
Disease graded subjectively from 0 to +++ (see text).

Table 6. Distribution of Marrow-Derived Cells

	Per Cent Fluorescent Cells			
Rat#	Joints	Bone Marrow	Lymph Nodes	Spleen
2	54	91	8	25
5	42	89	6	28
6	55	92	10	38
7	52	91	9	27
11	65	91	14	30
13	61	95	10	25
14	54	90	12	27
15	59	96	11	33
Mean	55.2	91.9	10.0	29.1
Standard deviation	± 6.9	±2.4	± 2.4	±4.5

the monocyte-histiocyte series 24-36%, and polymorphonuclears 3-9%. Typical cells of each type are shown in Figure 4. This result is consistent with earlier histologic descriptions of the lesions of adjuvant arthritis[5,9] in which there is the characteristic mononuclear infiltrate with a diffuse infiltration of polymorphonuclear cells superimposed in lesions of 1 or more days' duration.

Discussion

Lubaroff and Waksman[26] have demonstrated a marked variability in delayed tuberculin skin reactions in thymectomized, x-irradiated Lewis rats given allogeneic bone marrow prior to passive transfer of sensitized lymph node cells, and concluded that this variability in response was due to an incidence of homologous disease. When using F_1 bone marrow donors, they found more consistent results, since presumably the F_1 cells do not react to the host cells as foreign antigen. For this reason, F_1 bone marrow was used in the fluorescence assay group with adjuvant disease. As shown in the preliminary experiments, passive transfer of adjuvant disease was easily performed in (1) normal Lewis recipients, (2) thymectomized, irradiated Lewis rats receiving syngeneic bone marrow reconstitution, and

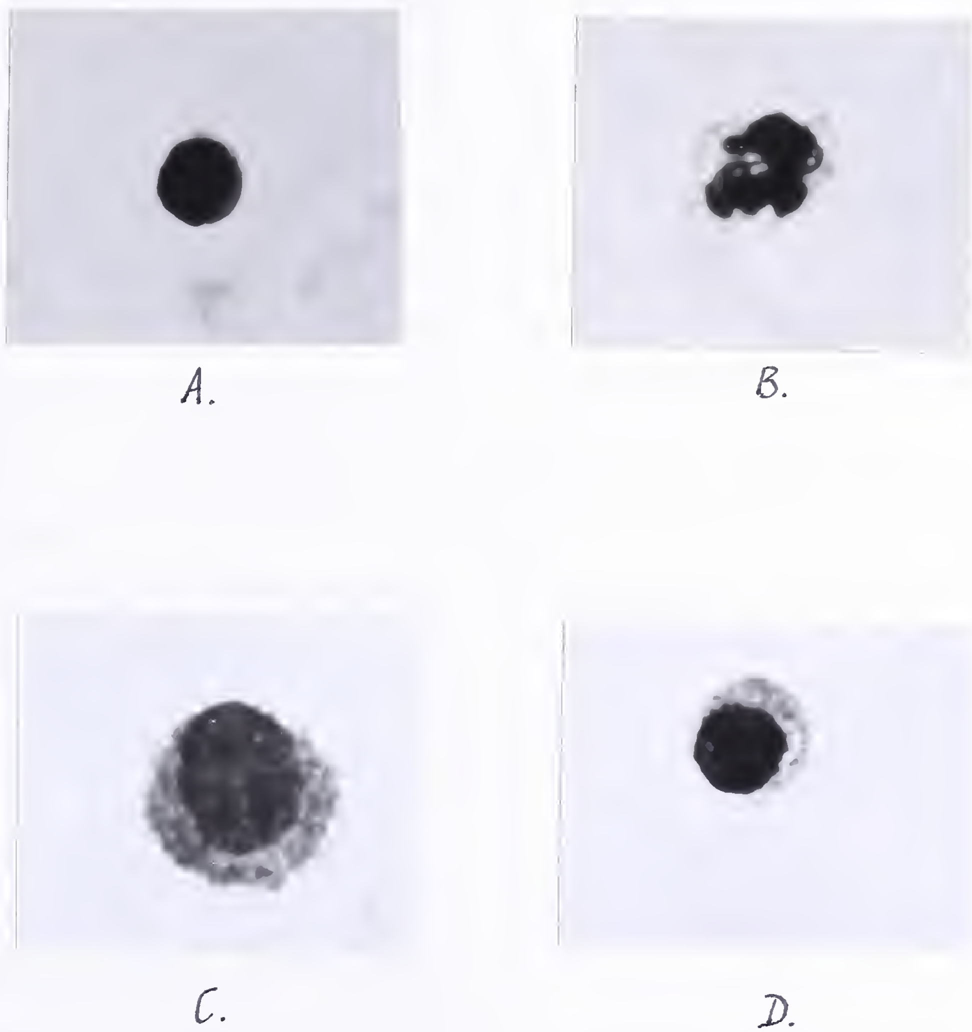


Figure 4. Examples of cell types commonly observed in Giemsa-stained smears of cells teased from affected joints. A. Lymphocyte. B. Polymorphonuclear. C. Histiocyte. D. Monocyte. (All x 1400).

(3) thymectomized, irradiated Lewis rats receiving F_1 bone marrow. Although there was no evident difference between the degree of passive disease as determined by onset and average intensity in the 3 groups of recipients (Tables 1-4), of the 2 irradiated groups of recipients, only those rats receiving F_1 bone marrow suffered 33% radiation deaths. Explanations could include the possibilities that although the doses used of syngeneic and F_1 bone marrow cells were approximately equal, they were not equally effective in restoring the bone marrow of the recipient rats or that there may have been an intercurrent infection in the farm at the time of the F_1 experiments, but no conclusion can be drawn.

Burstein and Waksman[10] using tritiated thymidine labeling techniques indicated that the cellular infiltrate in the lesions of adjuvant arthritis is derived from a rapidly replicating cell population in the blood or lymphoid tissues. By using the fluorescence labeling technique recently developed[29], it has been possible to label all of the progeny of the donor bone marrow cells instead of just recently divided cells, a limitation inherent in the tritiated thymidine technique. On the other hand, our system using thymectomized, x-irradiated, bone marrow reconstituted rats in which passive adjuvant disease has been induced with

"sensitized" lymph node cells cannot give results which reflect with certainty the situation which exists in active adjuvant disease. With this reservation in mind, our results suggest that the rapidly dividing precursors of the infiltrating cells in the lesions of adjuvant arthritis are from the bone marrow.

These results agree with evidence in other reactions of delayed hypersensitivity in which the infiltrating cells come from bone marrow precursors[24-26,28], and thus support earlier evidence[1-9] that the lesion of adjuvant arthritis has the delayed mechanism. Although only 24-36% of cells in smears from the arthritis lesions were of the monocyte-histiocyte series, and in other lesions of delayed hypersensitivity many more may be found[31], the percentages found of fluorescent cells in the assayed joints suggests there were more than those counted in the smears. The low monocyte-histiocyte counts on the smears may be due to the histologic similarity of monocytes to lymphocytes in the rat[22] and thus the figure of 55-73% lymphocytes found may be erroneously high.

The percentage of fluorescent cells in the bone marrow in our experiments was high enough that nearly all marrow-derived cells should have been fluorescent. The percentages found in involved joints were much higher than in the lymph nodes or spleen. This suggests

that a sizeable number of the infiltrating mononuclears come from the bone marrow. In addition, the absence of the thymus appears to exclude the possibility that these cells are processed in the thymus. Nevertheless, the latent period of several days leaves open the possibility that the cells differentiate somewhere outside the marrow, such as in the spleen. The low percentage of marrow-derived cells in the spleen could merely indicate that a sub-population only is involved. However, our results agree with earlier studies[26,28,29] in which the same technique was used to analyze tuberculin skin reactions, and with findings[24] using tritiated thymidine labeling in contact reactions in the guinea pig. The percentage of marrow-derived cells in the arthritis lesions, although a majority, was somewhat lower than the approximately 75% found for tuberculin skin lesions. This could be due to some inherent difference between the 2 systems or to the length of time between transfer and assay, which might allow a greater number of immunocompetent cells of the Lewis host to recover from x-irradiation. Nevertheless, in the joint lesions of adjuvant arthritis a majority of the infiltrating cells fluoresced, suggesting that they are, in the special circumstance of irradiated, passively sensitized animals, hematogenous and derived from bone marrow precursors.

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